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Targeted expression of HGF/SF in mouse mammary epithelium leads to metastatic adenosquamous carcinomas through the activation of multiple signal transduction pathways

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Overexpression of hepatocyte growth factor (HGF), also called scatter factor (SF), and its receptor c-Met are associated with poor prognosis for cancer patients. In particular, breast cancer cells can produce HGF that acts in a paracrine as well as in an autocrine manner. Therefore, HGF and c-Met are putative targets for cancer therapy. To explore HGF/c-Met signaling in breast cancer, we have generated transgenic mice expressing HGF specifically in mammary epithelium under the transcriptional control of the *whey acidic protein* (*WAP*) gene promoter. WAP-HGF transgenic females developed hyperplastic ductal trees and multifocal invasive tumors after several pregnancies, some of which progressed to lung metastases. Tumors produced HGF and displayed phosphorylated c-Met, which correlated with increased Akt as well as c-*myc* activation. A high growth rate, as demonstrated by Ki67 nuclear antigen staining, and a lack of progesterone receptor were characteristic of the tumors. Immunohistochemical analysis revealed areas of osteopontin (Opn) expression in WAP-HGF tumors and lung metastases in agreement with a previously reported role for Opn in invasive growth. We suggest that these mice may serve as a new breast cancer model for the evaluation of the effects of unscheduled HGF expression in breast cancer.
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Introduction

Hepatocyte growth factor (HGF) is a soluble, fibroblast-derived cytokine capable of stimulating motility, morphogenesis and proliferation of cells predominantly of epithelial or endothelial origin (reviewed in Zhang and Vande Woude, 2003). Target cells express c-Met, the high-affinity HGF receptor, and are located in close proximity to HGF-producing cells (Sonnenberg *et al*., 1993). The HGF/Met signaling pathway is active in vertebrates, where it regulates embryonic development (reviewed in Birchmeier and Gherardi, 1998) as well as tissue homeostasis in the adult (Yant *et al*., 1998; Wang *et al*., 2002; Yoshida *et al*., 2003). c-Met is a membrane tyrosine kinase receptor with pleiotropic activity, which contains docking sites for different proteins that are phosphorylated by the activated receptor. The activation of distinct signaling pathways elicits morphogenesis, mitogenesis and scattering of target cells (reviewed in Furge *et al*., 2000).

Deregulated HGF/Met signaling has been implicated in tumor progression towards an invasive-metastatic phenotype referred to as 'invasive growth' (Rong *et al*., 1994; Di Renzo *et al*., 1995, 2000; Takeuchi *et al*., 2003; Tolgay Ocal *et al*., 2003). The clinical importance of HGF/c-Met signaling in cancer resistance to antiangiogenic therapy has recently been identified. Observations point to c-Met expression as a molecular response to intratumor oxygen deprivation, indicating that c-Met may be responsible for the hypoxia-induced invasive switch (Pennacchietti *et al*., 2003). Inhibiting c-Met signaling, when targeting tumor angiogenesis, might prevent the potential spread of cancer cells (Pennacchietti *et al*., 2003).

The mammary gland originates during embryonic life, but mainly develops during puberty and acquires a functional differentiation with the onset of lactation (Hennighausen and Robinson, 2001). HGF is a morphogen in mammary development (Kamalati *et al*., 1999; reviewed in Pollard, 2001), and is expressed mainly in mammary stroma, whereas c-Met is found in the epithelial compartment (Niranjan *et al*., 1995). Experiments *in vitro* have shown that HGF induces ductal morphogenesis (Soriano *et al*., 1995; Yang *et al*., 1995; Niemann *et al*., 1998) and transplanted mammary epithelial cells transfected with a retrovirus expressing HGF in the cleared fat pad of host mice produced hyperplastic ductal trees (Yang *et al*., 1995). The expression of HGF under control of the metallothionein

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gene promoter in transgenic mice resulted in multiple developmental defects and predisposition to diverse neoplasias, including some in the mammary gland (Yant *et al*., 1998).

Tumor cells produce HGF that can act in either a paracrine or an autocrine manner (Rahimi *et al*., 1996; Edakuni *et al*., 2001). The elevated expression of HGF and c-Met has been observed in invasive ductal carcinomas from human breast cancers that have been associated with poor prognosis (Jin *et al*., 1997; Ghoussoub *et al*., 1998; Camp *et al*., 1999; Edakuni *et al*., 2001; Kang *et al*., 2003; Tolgay Ocal *et al*., 2003). Moreover, HGF and c-Met are considered possible markers for earlier recurrence and shorter survival in breast cancer patients (Yamashita *et al*., 1994; Kang *et al*., 2003). Further, osteopontin (Opn), a secreted glycophosphoprotein implicated in mammary gland differentiation and breast cancer (Nemir *et al*., 2000; Tuck and Chambers, 2001; Rudland *et al*., 2002), is an autocrine mediator of HGF-induced invasive growth (Medico *et al*., 2001).

These observations point to a role for HGF in mammary morphogenesis and breast cancer progression. However, the lack of transgenic mice in which the HGF/c-Met pathway is activated specifically in the mammary epithelium, precluded a molecular understanding of this signaling cascade in mammary gland physiology, cancer development and metastasis. Towards this goal, we have now developed transgenic mice in which HGF expression has been targeted to the mammary epithelium under the control of the *whey acidic protein* (WAP) gene promoter.

Materials and methods

Generation of transgenic mice overexpressing the HGF under the control of the WAP gene promoter

The mouse HGF cDNA sequence (a gift from Dr La Rochelle, NCI) was subcloned into the plasmid pUC198. This plasmid contained the last 20 bp of exon 2, the entire intron 2 and the first 50 bp of exon 3 of the *β-globin* gene, followed by a short polylinker in which the HGF cDNA was inserted. The rest of exon 3 of the *β-globin* gene (including the polyA addition signal) is located 3′ of the polylinker. In addition, the SV40 early polyA addition signal is present in this construct (Woodroofe *et al*., 1992). This plasmid was designated as pUC-HGF1. An *Sal*I–*Kpn*I fragment from the plasmid pUC-HGF1, containing the *β*-globin sequences, the HGF cDNA and the polyA addition signals, was inserted next to the *WAP* gene promoter in the WAP-SKII plasmid. This plasmid contained a *Bgl*II–*Kpn*I fragment of 1.6 kbp from the 3′ region of the *WAP* gene promoter. The resulting construct was the transgenic expression vector and was designated as pWAP-HGF. The construct was excised from the vector with *Eco*RV–*Asp*718, purified by agarose-gel electrophoresis and Elutip Affinity column® (Schleicher and Schuell), then resuspended at 2 ng/μl in 10 mM Tris (pH 7.4) 0.5 mM EDTA (Hogan, 1994). The pronuclear injection into FVB/N fertilized eggs was performed at the NIDDK transgenic facility.

Genomic DNA analysis

Transgenic founders were identified by Southern blot analysis of DNA from the tail tissue, digested with *Eco*RI. The entire mouse HGF cDNA was used as a probe. Transgenic offspring were subsequently identified by PCR using a set of primers specific for the transgene. Rabbit *β*-globin forward primer: 5′-TAC ACT GTT TGA GAT GAG G-3′ and HGF-ATG reverse primer: 5′-GAC TAG CTG CAG CAA CAG G-3′. The primers generated a single amplicon of 200 bp that was specific for the transgene.

RNA analysis

Total RNA was isolated and Northern blots were performed as described previously (Robinson *et al*., 1995). The whole mouse HGF cDNA or keratin 18 cDNA sequences were used as probes.

Histology, immunohistochemistry and immunofluorescence

Mammary tissue and tumors were harvested from recently euthanized mice. For whole mount examination, mammary tissues were fixed in Carnoy's solution for 4 h and stained with carmine aluminum overnight as described previously (Kordon *et al*., 1995). Whole mounts were embedded and sectioned using standard methods. For histological analysis, 5 μm paraffin sections were stained with H&E. For immunostaining, tissues were fixed for 4 h with paraformaldehyde and processed by standard protocols. Paraffin sections of 5 μm were deparaffinized and boiled in antigen retrieval solution (Vector), following the manufacturer's conditions, incubated overnight with anti-human-HGF, anti-Opn goat polyclonal antibodies at 20 μg/ml (R&D), phospho-AKT (Ser473) rabbit polyclonal antibody 1 : 100 dilution (Cell Signaling), anti-phospho-Myc (Thr58–Ser62) rabbit polyclonal antibody 1 : 100 dilution (Cell Signaling) or anti-human-PR rabbit polyclonal antibody 1 : 50 dilution (Dako #A0098). The primary incubation was followed by a 1 h incubation with the appropriate HRP-conjugated secondary antibody and 30 min with Vectastain ABC reagent (as per kit instructions). The slides were incubated for 2 min with the DAB developing solution. Immunostaining with mouse monoclonal Ki67 antibody (Novocastra) required an additional treatment with HCl previous to the antigen retrieval step. For immunofluorescence analysis, we performed the same tissue fixation, deparaffinization and antigen retrieval protocol as in the immunohistochemical analysis. We used anti-cytokeratin 1, 5 or 6 rabbit polyclonal antibodies (1 : 200 dilution, Babco) with anti-*β*-catenin mouse monoclonal (1 : 100 dilution, Transduction Laboratories) incubated during 1 h or anti-*β*-catenin with anti-hHGF (20 μg/ml dilution, R&D) incubated overnight. The secondary antibodies were Alexafluor 594-conjugated anti-rabbit and Alexafluor 488-conjugated anti-mouse or Alexafluor 594-conjugated anti-mouse and Alexafluor 488-conjugated anti-goat from Molecular Probes. Slides were observed using a Zeiss Axioscop equipped with filters for FITC, TRITC and FITC:TRITC.

Immunoprecipitation and Western blot

Immunoprecipitation reactions and Western blotting were performed as described previously (Liu *et al*., 1996). Anti-mouse c-Met (R&D) at 0.2 μg/ml for WB and 4 μg/ml for IP; phosphotyrosine (4G10 from Upstate Biotechnology) at 1 : 5000 for WB.

Results

Generation of WAP-HGF transgenic mice

The consequences of unscheduled HGF expression in the mammary epithelium *in vivo* were explored in transgenic mice that carry a HGF cDNA under the control of the *WAP* gene promoter (Figure 1a). The activity of this promoter is largely confined to differentiating mammary alveolar epithelium (Pittius *et al*., 1988). A total of 18 WAP-HGF transgenic founder mice were generated by pronuclear injection, and 16 lines were established. The expression of the transgene in mammary tissue from lactating mice was analysed by Northern blot and immunohistochemistry. Line 402 exhibited the highest levels of transgenic HGF mRNA (Figure 1b) and immunohistochemical analysis confirmed an excess of HGF in the mammary alveolar epithelium (Figure 1c). A lower expression was detected in other lines (data not shown). No HGF staining was detected in the alveoli of wild-type lactating littermates (Figure 1d).

Hyperplastic mammary ductal trees develop in WAP-HGF virgin mice

Evidence that an excess of HGF adversely affects mammary development came from virgin females that presented hyperplastic ductal trees, which were characterized by the appearance of abundant lobular structures protruding from ducts (Figure 2a and c, arrows). Such lobular structures were absent from the mammary ducts in wild-type littermates (Figure 2b). During lactation, large and normal appearing lobuloalveolar units developed in WAP-HGF mice, masking the aberrant lobular structures observed in virgin transgenic mice (not shown). However, hyperplastic ductal trees were again evident upon involution (not shown). Another alteration consistently observed in WAP-HGF virgin females was the presence of a thick layer of fibroblasts surrounding the mammary epithelium (Figure 2c), as compared with wild-type littermates (Figure 2d). Immunohistochemical analysis confirmed a mosaic expression of HGF in ducts from transgenic virgin females (Figure 2e, arrow), while no expression was detected in wild-type females (Figure 2f).

Development of fast-growing carcinomas and metastases after several pregnancies

The *WAP* gene promoter, although active during estrus, is prominently induced during pregnancy and remains active throughout lactation. Consistent with this, the continuous expression of HGF through successive pregnancies resulted in the appearance of mammary carcinomas. Out of a cohort of 55 mice observed for a period of 10 months, while continuously breeding, 49 developed visible masses (Figure 3a). Mice were killed 4–8 weeks after the tumors became visible and reached a diameter of about 15 mm. Most mice developed multifocal tumors in more than one gland and macroscopic lung metastases were found in 12 of the 55 mice. These

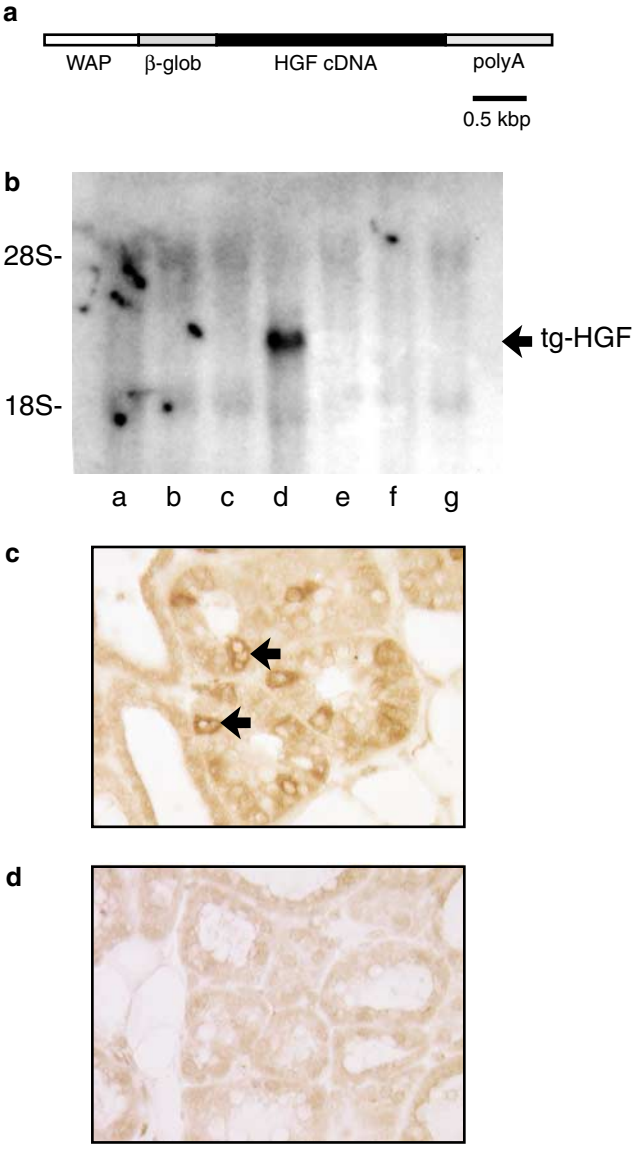


Figure 1 (**a**) Schematic representation of the transgene construct. Mouse HGF cDNA was inserted into a plasmid containing the second intron (*β*-glob) and polyA addition signal (polyA) of the *β-globin* gene, in addition to the SV40 early polyA sequences. This construct was placed downstream of the *Bgl*II–*Kpn*I fragment (1.6 kbp) of the *WAP* gene promoter (WAP) to generate the WAP-HGF transgenic construct. (**b**) Total RNA Northern blot analysis of transgenic HGF (tg-HGF) expression in mammary tissues from different transgenic lines at lactation day 1. Lane d corresponding to transgenic line 402 presents a noticeable band of tg-HGF expression. The location of the bands corresponding to the ribosomal RNAs is marked by18S and 28S. No expression of endogenous HGF was detected. (**c** and **d**) Immunohistochemical analysis of the HGF protein in mammary tissues from a transgenic female (**c**) and a wild-type female (**d**) at lactation day 1. High expression is detected in some of the alveolar cells in panel c (arrows), while only uniform background staining is detected in panel d

12 mice had multiple large mammary tumors. Out of a total of 50, 10 virgin females also developed one or multiple tumors before 1 year of age. The lower incidence of tumors in virgin females is consistent with

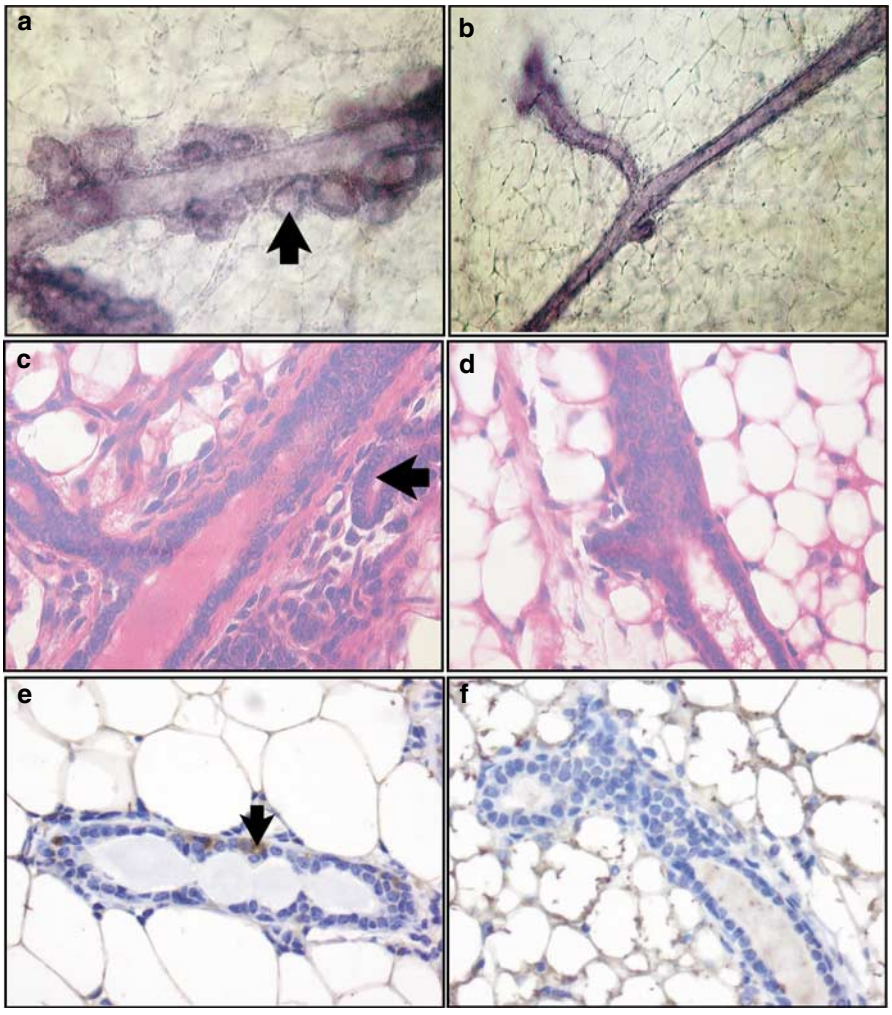


Figure 2 Whole-mount, histological and immunohistochemical analysis of WAP-HGF (**a**, **c** and **e**) and wild-type (**b**, **d** and **f**) virgin mammary ducts. (**a** and **b**) Carmine aluminum-stained WAP-HGF (**a**) and wild-type (**b**) mammary ducts, at 7 weeks of age. The ducts in WAP-HGF transgenic virgin females present abundant lobular epithelial structures (**a** and **b**, arrows), while wild-type ducts show a smooth surface (**b**). Histological analysis of the whole mounts shown in (**a** and **b**) revealed a hyperplastic layer of fibroblasts surrounding the ducts in WAP-HGF transgenic virgin mammary tissue (**c**) as compared to the virgin wild-type histological section (**d**). (**e** and **f**) Immunohistochemical analysis of the HGF protein in mammary tissues from a transgenic virgin female (**e**) and a wild-type virgin female (**f**) at 12 months of age. HGF is detected in the cytoplasm of some of the ductal cells in panel e (arrow), while no protein is detected in panel f. Magnification in panels a and b is $\times 200$, in c and d is $\times 650$ and in e and f is $\times 400$

the transcriptional induction of the *WAP* gene promoter during pregnancy.

Histological analyses of WAP-HGF tumors revealed a complex glandular (Figure 3c, arrowhead) and squamous pattern (Figure 3c, arrow) with extensive necrosis. The glandular tissue was found in large and small nodules separated by delicate strands of stroma (Figure 3d). The nodules contained sheets of poorly organized small glandular units surrounded by a peripheral palisade of cells. Many of the central cells had clear round fat vacuoles (Figure 3d, arrow) imparting a secretory pattern. Small foci of squamous cells with central extracellular keratin were admixed with the glandular elements (not shown). Large areas of squamous differentiation (Figure 3c, arrow) were also present forming large cystic spaces filled with laminar keratin. Tumors presented large areas of high growth rate as assessed by nuclear staining after immunohistochemical analysis with anti-Ki67 antibody (Figure 3b), a marker of cycling cells.

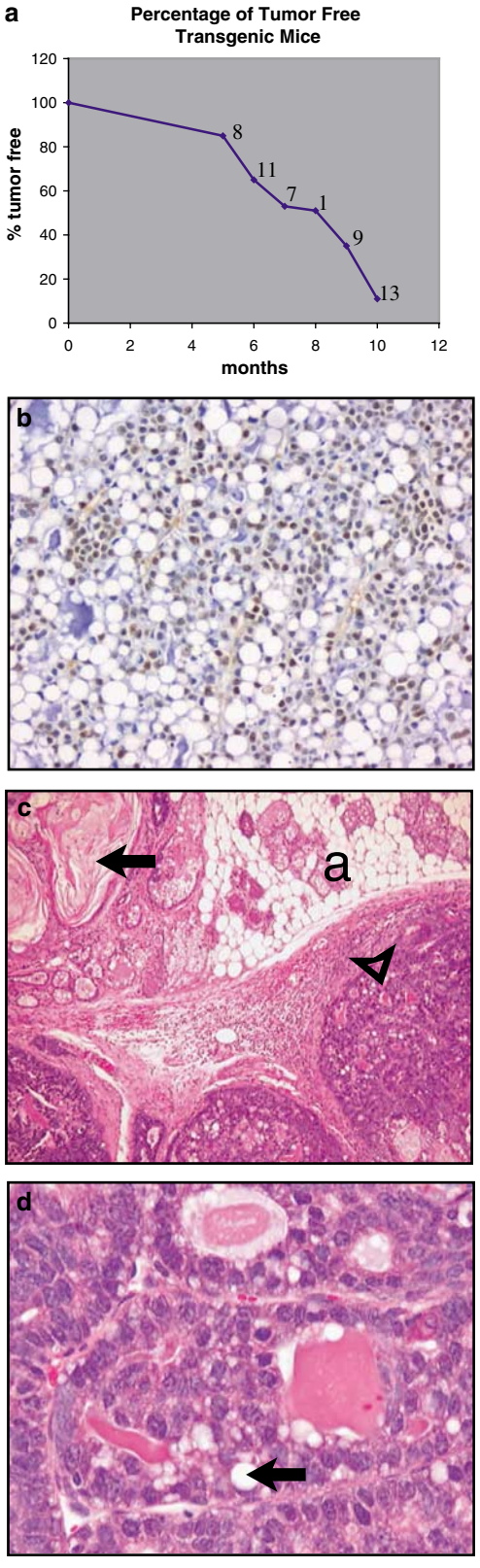
WAP-HGF tumors expressed HGF and displayed c-Met phosphorylation

Histological (Figure 4a) and immunohistochemical analyses (Figure 4b) of serial sections confirmed high levels of HGF in areas of adenocarcinomas (Figures 4a and b, arrowhead) that were surrounded by areas of squamous cells (Figure 4a and d, arrow).

Upon HGF binding, the c-Met receptor is tyrosine phosphorylated.

Immunoprecipitation of c-Met followed by phosphotyrosine blotting revealed higher levels of c-Met phosphorylation in mammary tissue from WAP-HGF

lactating mice (Figure 4d, lanes b and d) or WAP-HGF tumors (Figure 4d, lanes c, e and f) as compared to the corresponding wild-type tissue (Figure 4d, lanes a and g). This demonstrates that the c-Met signaling



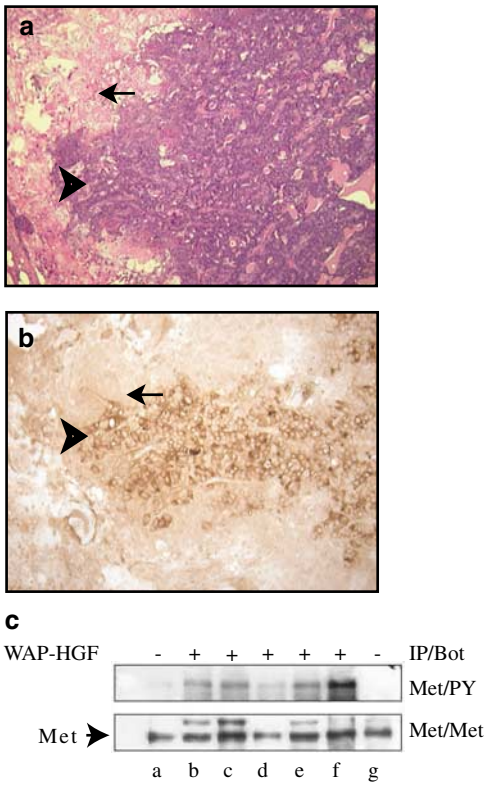


Figure 4 (**a** and **b**) Histological and immunohistochemical analyses of a WAP-HGF tumor. The histological analysis (**a**) shows areas of adenocarcinoma (arrowhead) and areas of necrotic squamous metaplasia (arrow). A serial section from the tumor in panel a was analysed for HGF expression using an anti-human HGF polyclonal antibody (**b**) Abundant staining is detected in the adenocarcinoma (arrowhead) and no staining is detected in the area of squamous metaplasia (arrow). The magnification of the pictures is $\times 200$. (**c**) Analysis of c-Met protein activation in WAP-HGF lactating tissues and tumors. Lactating mammary tissue (a, b, d and g) and tumor tissue (c, e and f) lysates were immunoprecipitated with a c-Met polyclonal antibody. Western blots with antiphosphotyrosine (pY) (upper) or anti-c-Met (lower) were performed. (a) Mammary gland protein lysate from wild-type female at lactation day 15. (b) Mammary gland protein lysate from a WAP-HGF transgenic female at lactation day one and (c) tumor tissue protein lysate from the same mouse. (d) Mammary gland protein lysate from mouse WAP-HFG at lactation day 18 and (e) tumor tissue protein lysate from the same mouse. (f) Tumor tissue protein lysate from a WAP-HGF virgin female. (g) Mammary gland protein lysate from a wild-type female at lactation day 1

Figure 3 (**a**) Tumor development in transgenic mice. Out of a cohort of 55 female mice from line 402 observed for a period of 10 months while continuously breeding, only six females remained tumor free at 10 months of age. The numbers 8, 11, 7, 1, 9 and 13 indicate the number of animals that developed tumors at each time point. (**b**) Immunohistochemical detection of Ki67 protein in a WAP-HGF tumor section. The brown-colored nuclei shown in **b** demonstrate Ki67 expression, indicating cell proliferation. Magnification in panel b is $200\times$. (**c**) Histological analysis of a papillary and secretory keratoacanthoma and surrounding mammary tissue from a 36-week old WAP-HGF lactating female. The picture shows areas of glandular tissue (arrowhead) surrounded with fibrous connective tissue, areas of squamous differentiation forming large cystic spaces (arrow) and normal secreting alveoli (**a**). The magnification of this picture is $\times 100$. (**d**) A higher magnification of an area of glandular tissue within the tumor. The arrow points to a clear round fat vacuole, imparting a secretory pattern. The magnification of this picture is $\times 650$

pathway is activated in mammary tissue and tumors from WAP-HGF transgenic mice.

c-myc *and Akt were activated in WAP-HGF tumors*

To achieve a molecular understanding of HGF-mediated mammary tumorigenesis, we characterized potentially oncogenic downstream mediators of c-Met signaling. The proto-oncogene c-*myc* encodes a transcription factor, which plays a major role in the regulation of normal cellular proliferation and is aberrantly expressed in many breast tumors. c-*myc* can be activated by the MAP kinases, which are downstream mediators of growth factor receptors, like c-Met. Immunohistochemical analysis of phospho-c-*myc* (Thr58)–(Ser62), the activated form of c-*myc*, revealed extensive nuclear staining in the tumors (Figure 5b), but not in adjacent normal mammary tissue (Figure 5a), indicating that myc was activated during tumorigenesis in WAP-HGF transgenic mice. No staining was detected in the control slides, when the first antibody was omitted (Figure 5e and f).

Akt is a downstream mediator of c-Met, since c-Met activation causes Akt phosphorylation (reviewed in Furge *et al*., 2000). Immunohistochemical analysis of phospho-Akt (Ser473) demonstrated that Akt was preferentially activated in the tumor front (Figure 5d, arrow) and localized within the nuclei. Akt activation was low in normal surrounding mammary tissue as shown by the sparse nuclear staining (Figure 5c). No

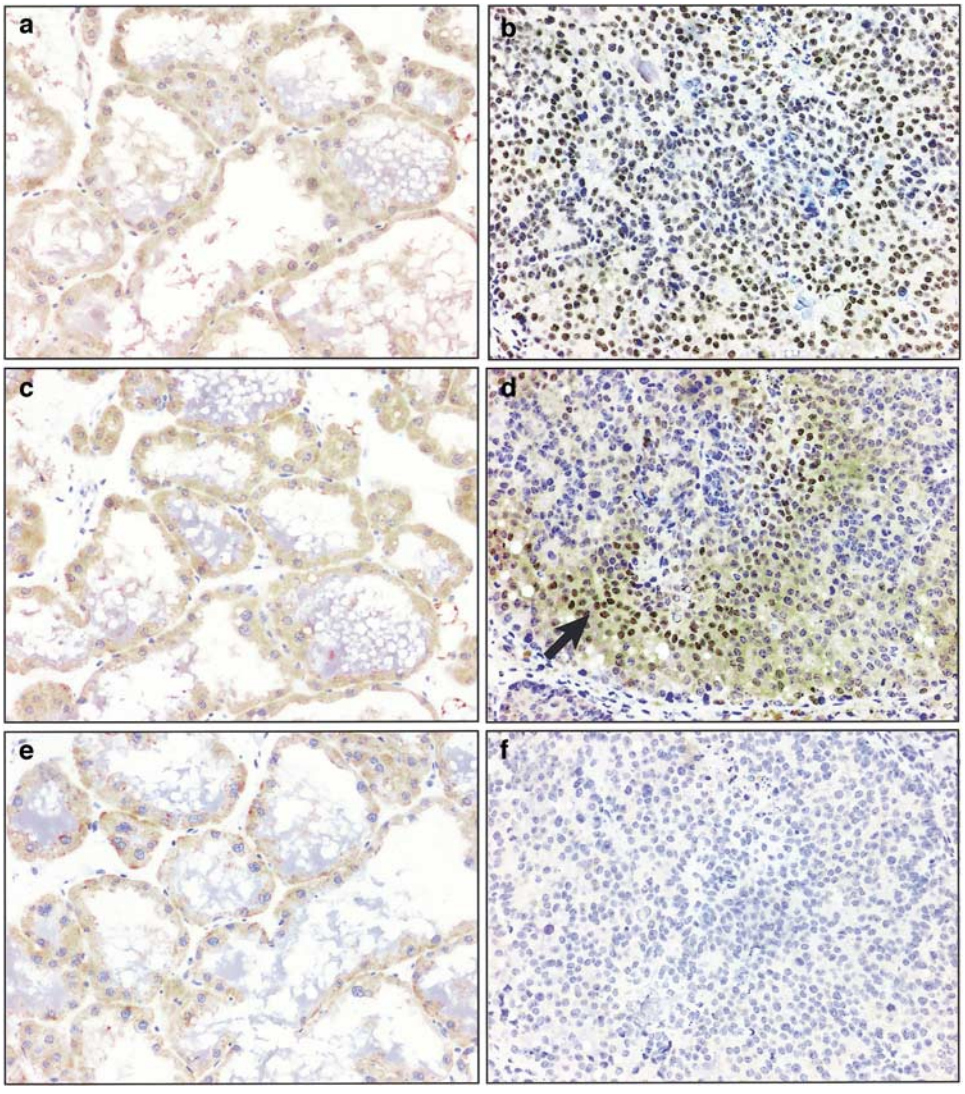


Figure 5 Immunohistochemical analyses of phospho-myc (**a** and **b**) and phospho-AKT (**c** and **d**) in lactating (**a**, **c** and **e**) and tumor (**b**, **d** and **f**) tissues from WAP-HGF mice. No staining was detected when the samples were incubated in the absence of primary antibody (**e** and **f**). AKT and c-Myc are preferentially activated in the tumor (**b** and **d**) in contrast to normal tissue (**a** and **c**). (**a** and **b**) Phospho-myc rabbit polyclonal antibody stains a higher proportion of nuclei in the tumor (**b**) than in normal tissue (**a**). (**c** and **d**) Phospho-AKT rabbit polyclonal stains sparse nuclei in the normal tissue (**c**), while many dark nuclei are detected in the tumor (**d**, arrow). Magnification of all panels was $\times 200$

nuclear staining was detected in normal tissue and tumor control slides (Figure 5e and b) when the primary antibody was omitted from the reaction.

WAP-HGF tumors presented malignancy markers

Progesterone receptor (PR) deficiency is associated with activated growth factor signaling in breast cancer cells and with aggressive tumor phenotypes. We analysed the presence of PR in WAP-HGF tumors and in surrounding tissues. Immunohistochemistry demonstrated a mosaic PR staining in highly differentiated parts of the tumor (Figure 6a, arrow) similar to the staining observed in wild-type mammary tissue (not shown). In contrast, the undifferentiated regions within the tumor tissues presented no PR staining (Figure 6c). No staining was detected in control slides, when the primary antibody was omitted (Figure 6b and d).

Opn is a secreted matrix protein implicated in HGF-mediated invasive growth. Immunohistochemical analysis of WAP-HGF tumors demonstrated abundant Opn expression localized within areas of the mammary tumor tissue (Figure 6e, arrow) and in WAP-HGF lung metastases (Figure 6g). No staining was detected in control slides when the primary antibody was omitted from the incubation (Figure 6f and h).

Accumulation of β*-catenin in areas of squamous metaplasia*

Stabilization of β-catenin in the mammary epithelium of transgenic mice results in the appearance of squamous metaplasia (Gallagher *et al*., 2002; Miyoshi *et al*., 2002). Profuse squamous metaplasias found in WAP-HGF tumors (Figure 7a, arrows) and lung metastasis (Figure 7b, arrow) prompted us to investigate whether β-catenin was also stabilized in cells from WAP-HGF tumors. Only membrane-bound β-catenin was detected in the normal mammary epithelium and most adenocarcinomas. However, both cytoplasmic and nuclear β-catenin was detected in large clusters of cells surrounding the areas of squamous metaplasias in WAP-HGF tumors (Figure 7c). This accumulation of β-catenin was also evident in the metastases and microscopic squamous metaplasias present in the WAP-HGF mammary glands (not shown). Areas of squamous metaplasias are characterized at the molecular level through the expression of epidermal-specific keratins, such as keratin 1 (not shown) and keratin 6 (Figure 7e), which are not expressed in normal adult mammary tissue. A cluster of tumor cells presenting nuclear and cytoplasmic staining of β-catenin (Figure 7e, arrowhead) were located in close proximity to cells presenting keratin 6 in their cytoplasm (Figure 7e). These data are in agreement with a role for HGF in β-catenin stabilization that has been previously reported (Monga *et al*., 2002). Immunofluorescence staining with anti-β-catenin and anti-HGF (Figure 7f) antibodies identified clusters of cells demonstrating an accumulation of β-catenin (Figure 7f, arrowhead) adjacent to the cells presenting abundant HGF protein (Figure 7f, arrow). This may point to a paracrine or indirect role for HGF in the observed β-catenin accumulation.

Discussion

Many studies have correlated HGF/c-Met signaling in breast cancer with a poor prognosis for the patient (Yamashita *et al*., 1994; Di Renzo *et al*., 1995; Jin *et al*., 1997; Camp *et al*., 1999; Edakuni *et al*., 2001; Elliott *et al*., 2002; Greenberg *et al*., 2003; Kang *et al*., 2003; Tolgay Ocal *et al*., 2003). Nevertheless, c-Met activating mutations are rare in breast cancer (Bieche *et al*., 1999). While normal mammary epithelial cells do not express significant amounts of HGF, breast cancer cells can be major producers of HGF that signals in a paracrine and autocrine manner (Rahimi *et al*., 1996; Tuck *et al*., 1996).

Transgenic mice that express HGF under the control of the hormone-inducible *WAP* gene promoter (WAP-HGF mice) developed tumors that appeared almost simultaneously in multiple glands after several cycles of transgene induction through pregnancy and lactation. Both tumor tissue and surrounding mammary tissue expressed HGF and presented c-Met receptor activation. Therefore, breast tumors in WAP-HGF mice resemble HGF overexpression-mediated c-Met activation found in some human breast cancers.

WAP-HGF tumors presented characteristics of breast tumors with an aggressive phenotype, namely a high growth rate, as demonstrated by the Ki67 labeling index and the loss of PR expression. Furthermore, c-*myc* and Akt activation are increased in WAP-HGF tumors as compared to the surrounding lactating mammary tissue, suggesting that the activation of both pathways contributes to tumor formation. Akt and c-*myc* are downstream effectors of c-Met (Bowers *et al*., 2000; Furge *et al*., 2000; Michaelson and Leder, 2001; Xiao *et al*., 2001; Walter *et al*., 2002). C-*myc* and Akt gain of function are often associated with aggressive phenotypes in breast cancer (Aulmann *et al*., 2002; Liang *et al*., 2002; Schlotter *et al*., 2003; Stal *et al*., 2003). While the constitutive activation of Akt may be responsible for the loss of PR expression in breast cancer and is associated with multidrug resistance (Cui *et al*., 2003; Knuefermann *et al*., 2003), c-*myc* overexpression is associated with genomic instability (Vafa *et al*., 2002). Both signaling events could contribute to the fast growth and aggressive phenotype in WAP-HGF tumors.

Macroscopic lung metastases were found in 22% of the killed WAP-HGF transgenic mice, supporting a role for HGF in metastatic growth (reviewed in Elliott *et al*., 2002). Elevated levels of the secreted matrix protein Opn have been found in tumors and blood of patients with metastatic breast cancer, offering the potential to function as a prognostic marker (Rittling and Novick, 1997; Tuck and Chambers, 2001; Rudland *et al*., 2002). A role for Opn in mammary cell migration was linked to c-Met activation (Tuck *et al*., 2000) and Opn has been

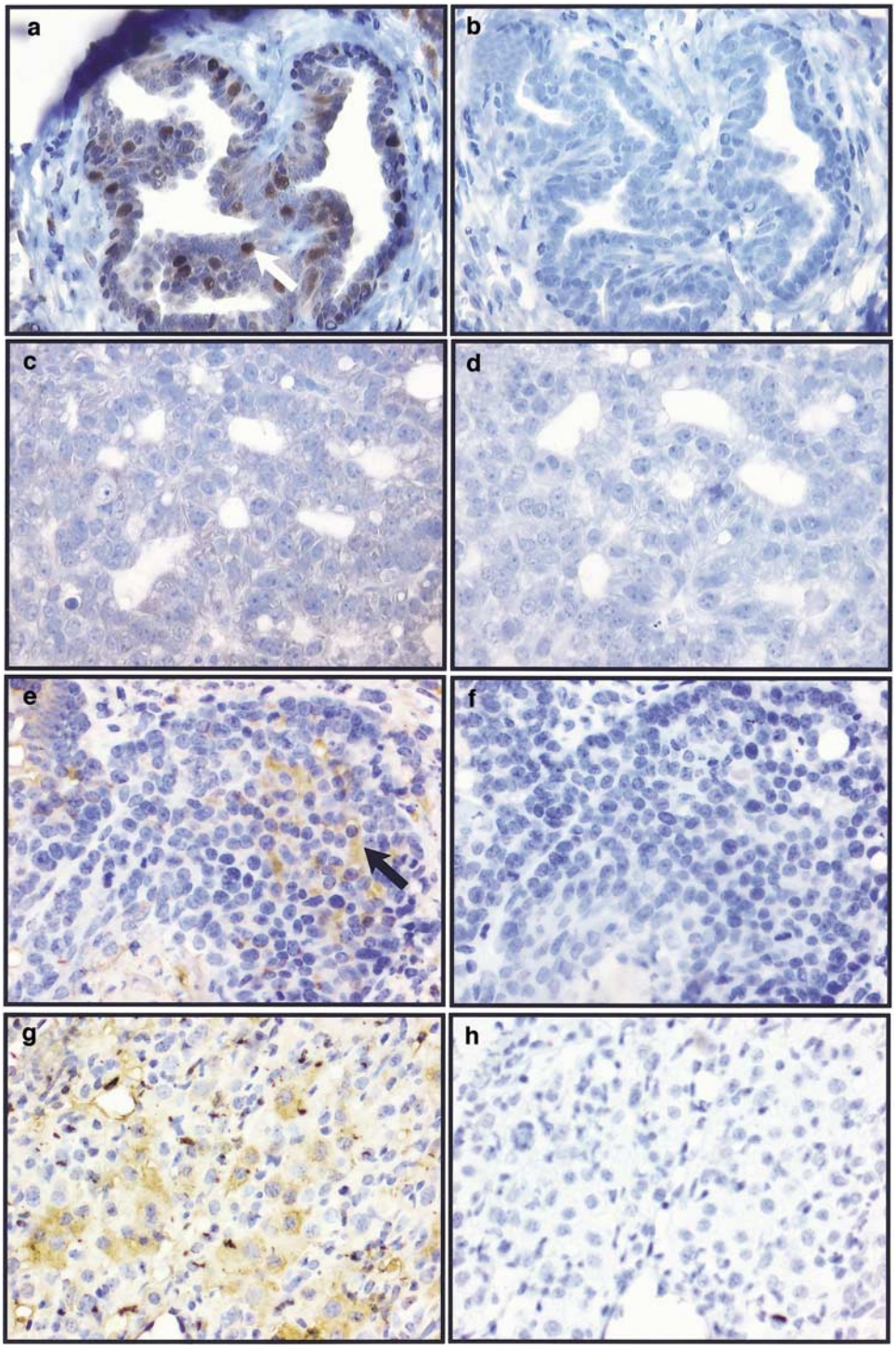


Figure 6 Markers of a malignant phenotype. Immunohistochemical analyses of PR (**a** and **c**) and osteopontin (**e** and **g**) in WAP-HGF tissues. No staining was detected when the primary antibody was not present in the incubation reaction (**b**, **d**, **f** and **h**). PR was detected in the nuclei of cells from premalignant lesions (**a**, arrow), but was not detected in less-differentiated tumors (**c**). Osteopontin (Opt) staining was detected in mammary tumor sections (**e**, arrow) in addition to sections from a lung metastasis (**g**)

defined as an autocrine mediator of HGF-induced invasive growth (Medico *et al*., 2001). This study demonstrates the high focal expression of Opn in WAP-HGF tumors and metastases. The finding that Opn is expressed in WAP-HGF metastases supports previous findings that associate Opn overexpression with metastatic growth (Oates *et al*., 1996).

WAP-HGF mammary tumors presented areas of squamous metaplasias, which were characterized by the nuclear accumulation of β-catenin and aberrant

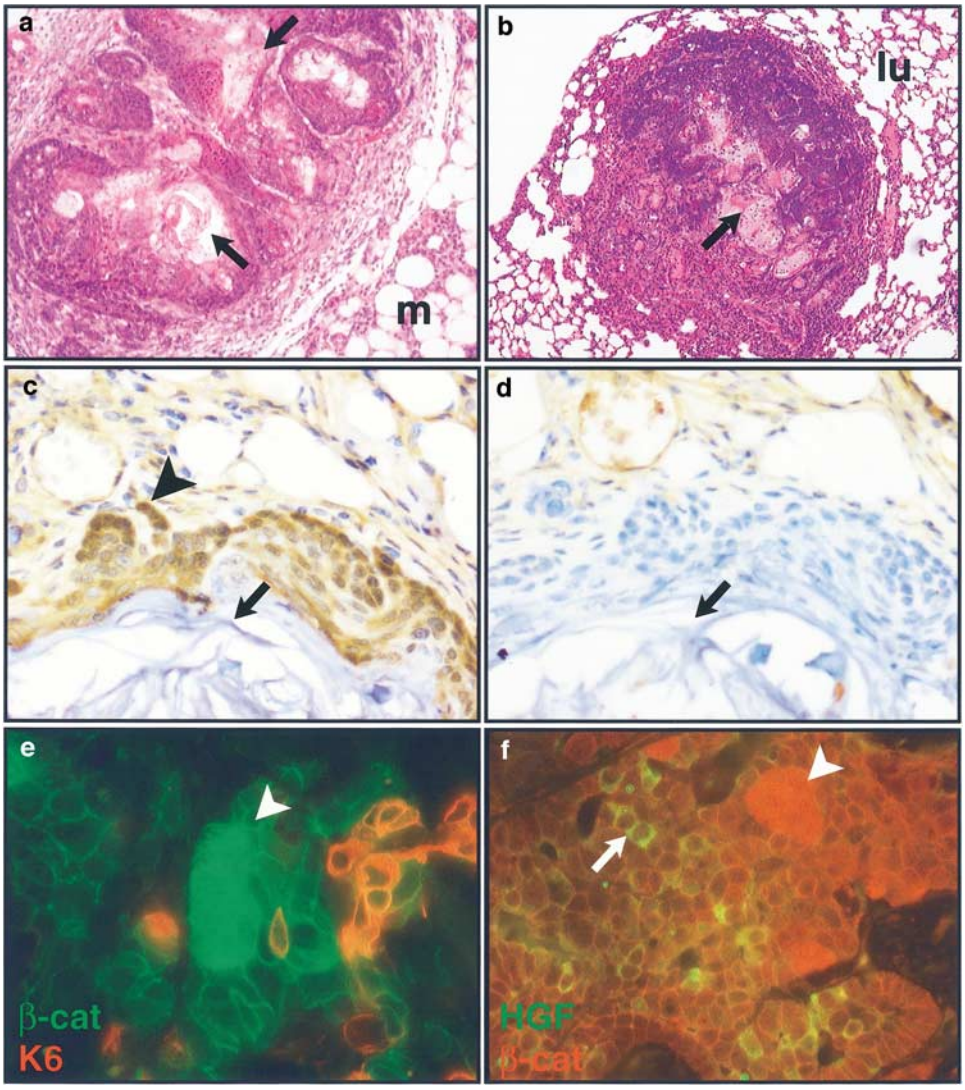


Figure 7 Immunohistological characterization of the squamous metaplasias in WAP-HGF tumors. (**a**) H&E staining of a characteristic squamous lesion (arrows) and normal mammary tissue (m) surrounding the lesion. (**b**) H&E staining of a lung metastases from an 8-month WAP-HGF transgenic female bearing several prominent primary tumors. A large area of squamous metaplasia is visible in the metastasis (arrow). Normal lung tissue (lu) surrounds the tumor. (**c** and **d**) Immunohistochemical characterization of β-catenin staining in a WAP-HGF tumor. (**c**) Tumor section presenting β-catenin staining in the nuclei and cytoplasm of cells (arrowhead) surrounding an area of squamous metaplasias (arrow). (**d**) No staining was detected in serial sections when the primary antibody was absent in the incubation reaction. (**e** and **f**) Immunofluorescence analysis of β-catenin staining in a WAP-HGF tumor. (**e**) Tumor section presenting membrane-bound -catenin (green) in all tumor cells with cytoplasmic and nuclear -catenin in a cluster of tumor cells (white arrowhead). Cytokeratin 6 (red), not expressed normally in the mammary gland, serves as a marker of squamous transdifferentiation. The amplification was $\times 1000$. (**f**) Mosaic pattern of HGF (green, arrow) in a WAP-HGF tumor. In close proximity to the HGF expressing a cluster of cells presenting cytoplasmic and nuclear β-catenin (red, arrowhead). Magnification was $\times 650$

keratin expression. A role for β-catenin in squamous metaplasia induction has been described in two mammary-specific transgenic mouse systems that resulted in stabilized β-catenin (Gallagher *et al*., 2002; Miyoshi *et al*., 2002). In addition, the formation of squamous metaplasias in response to β-catenin accumulation has been described in transgenic mice that overexpress the Wnt pathway proteins in mammary tissue (Rosner *et al*., 2002). Other authors have found a relation between c-Met activation and β-catenin stabilization in cultured cells (Danilkovitch-Miagkova *et al*., 2001; Monga *et al*., 2002). The c-Met/β-catenin relationship has also been described in colorectal carcinomas (Boon *et al*., 2002) and invasive breast carcinoma immunohistological samples (Nakopoulou *et al*., 2000). Our results demonstrate an accumulation of β-catenin in clusters of cells surrounding areas of squamous metaplasia in WAP-HGF tumors, and thus suggest a relationship between the HGF/c-Met and Wnt/β-catenin pathways. The finding that HGF and β-catenin accumulate in different cells points to a paracrine effect.

The latency period in WAP-HGF mammary carcinogenesis points to the requirement for additional mutations in order to initiate tumorigenesis. The possibility that HGF plays a role in tumor progression as opposed to tumor initiation would be in agreement with the reported role for HGF in the progression of malignant human breast cancers. Breeding WAP-HGF mice with other mammary tumor models will be instrumental in further determining the role for HGF/c-Met signaling in malignant breast cancer progression.

Alterations in normal mammary gland development were also found in WAP-HGF virgin female mice. Mammary ductal trees presented an altered morphology characterized by a large number of protruding structures resembling aborted branches covering the ductal surface. This phenotype could be attributed to an excess of the HGF protein found in isolated cells within the WAP-HGF virgin mammary ducts (Figure 2e). Although a higher HGF expression was detected in the lobuloalveolar structures of lactating glands (Figure 1c), no morphologic or functional alterations were observed during pregnancy or lactation. These observations point to a role for HGF/c-Met signaling in branching morphogenesis and not in lobuloalveolar proliferation, in agreement with previous *in vitro* data (Niemann *et al*., 1998).

Here, we report the generation and characterization of a mouse model that can be used to evaluate the specific effects of HGF expression in breast cancer. The histological and molecular lesions found in these mice corroborate the hypothesized role for HGF with respect to invasive breast cancer. Recent data have pointed to the therapeutic necessity of inhibiting c-Met signaling when targeting tumor angiogenesis in order to prevent the hypoxia-induced invasive switch (Pennacchietti *et al*., 2003). The WAP-HGF mouse model will be a useful tool for testing therapeutic drugs designed to block this signaling pathway in breast cancer.

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